## REMARKS

Claim 3 has been amended to correct the typographical error kindly pointed out by the Examiner. The claim also has been modified to give the proper chemical name of TRIMID appearing on page 5 of the specification. Lastly, "the" has been stricken from line 2 of that claim.

The claims before the Examiner are claims 3 and 6 to 10.

The amendment to claim 3 discussed above is believed to overcome the objection thereto.

The rejection of claims 3 and 6 under 35 USC 103 as unpatentable over Swan et al. '056 or Hubbell et al. '914 in view of Chai-Gao et al. '802 is respectfully traversed.

Applicants submit with respect that the arguments presented to date in support of patentability of the claims are proper ones and establish patentability over the cited art; we provide the following additional arguments.

The references, absent the template provided by applicants' specification and claims, do not properly teach or suggest the combination of those references in a way to lead to the claimed subject matter. The Examiner again asserts that the instant claims merely require co-immobilization of a TRIMID protein and

4

dextran and, as such, Swan et al. '056 teaches these aspects of the invention. Applicants respectfully disagree because claim 3 (the only independent claim) states absolutely that "the dextran is attached to the carrier" and that attachment is "through a component resulting from the irradiation of the 3-trifluoromethyl-3-(m-isothiocyanophenyl)-diazirine (TRIMID)-modified aminodextran." Thus, the claim specifies how the dextran is attached to the carrier. Moreover, and no less importantly, the connection is not through the TRIMID-modified amino dextran but through "a component resulting from the irradiation of" that material. The references therefore do not properly teach or suggest the invention claimed here. The Examiner has extrapolated the teachings of the prior art and drawn conclusions therefrom in a manner not justified by the prior art.

Applicants moreover point out that Swan et al. '056 at column 11, lines 52 to 61, discusses the nature of the film surface that is to be covalently bonded by latent reactive groups of the coupling compound. It is stated at column 11, lines 58 to 61, that the surfaces "preferably have abstractable hydrogen atoms and participate readily in the formation of

covalent bonds upon activation of the latent reactive groups."
Forming covalent bonds involving hydrogen abstraction is
applicable for photopolymerization initiated by
photointermediates (ketyl) radicals generated, for instance, by
benzophenone analogs which initiate propagating polymerization
reactions in accordance with the following scheme.
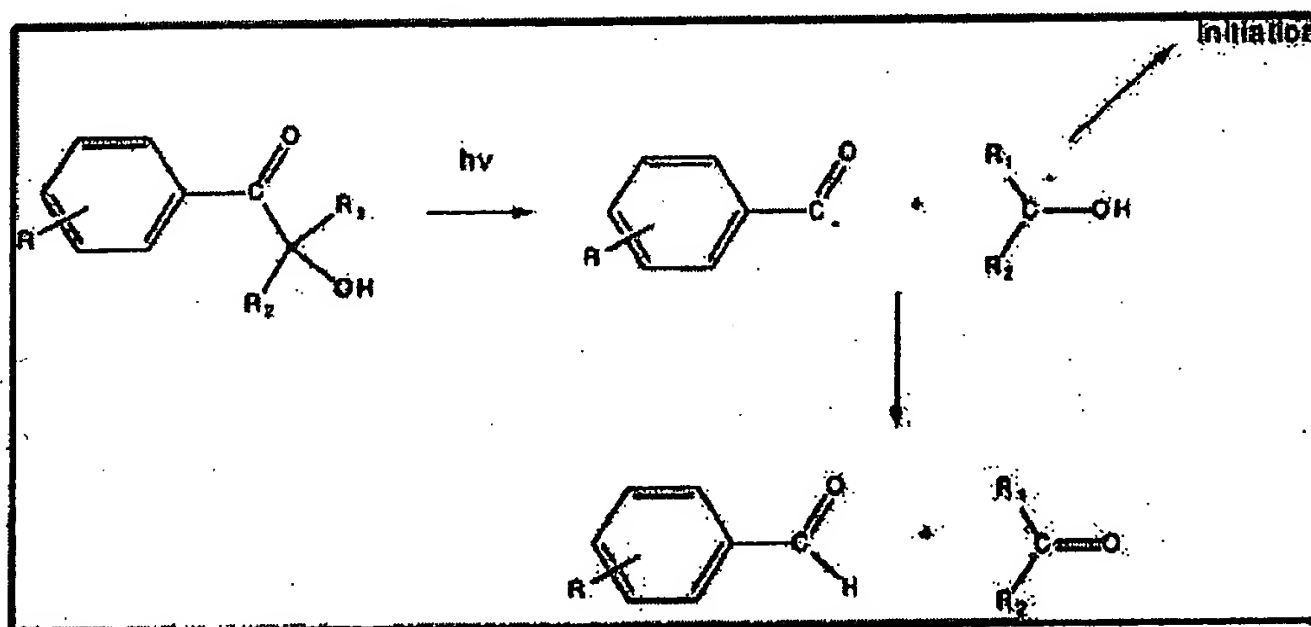


Applicants' invention uses reactive carbene intermediates
generated from aryl diazarines. Carbenes form new covalent
bonds by insertion into existing covalent bonds and do not
initiate propagating polymerization reactions.

6

Carbenes undergo insertion reactions while the photoproducts of benzophenones bind to the materials by radical mediated processes, e.g., a generated ketyl, radical may bind to a radical species formed on the surface material after hydrogen atom abstraction. With this step, new radicals are formed and polymerization continues until there is no more substrate providing abstractable hydrogen atoms. Covalent bonds are formed by radical to radical coupling. Such knowledge was known at the time of the Swan et al. '056 invention; see the enclosed copy of Optical Engineering (1995) 34-2339 to 2346.

As pointed out in previous replies, Swan et al. '056 refers to dextran sulfate but not dextran. The claims have been amended to make it apparent that dextran is used in the present invention and it is believed clear that Swan et al. '056 distinguished between dextran and dextran sulfate. Thus the reference does not properly teach or suggest the use of dextran.

The Examiner has stated that Swan et al. '056 discloses that dextran may be the coupling compound but applicants disagree. The reference lists various coupling compounds in claim 7 but neither dextran nor dextran sulfate appear. In contrast, both claim 2 and claim 5 of the patent list dextran

7

sulfate as either as a first (trapped) or a second (diffusible) chemical species. One reading the patent without instant claim 3 as a point of reference would not find the patent to teach what it is asserted to teach. The rejection should be withdrawn.

The general teachings of Hubbell et al. '914 do not provide a proper base for the rejection. The Examiner is again directed to applicants' previous arguments explaining how the artisan cannot properly be directed to the subject matter claimed here. The listing of dextran as a main portion of a derivative functioning as a macromer and the discussion of photoinitiators (see particularly the discussion of photoinitiating dyes in the last paragraph of column 11) doesn't put one on track to practice applicants' invention.

Applicants again say with respect that the Examiner, despite the disclaimer at 8 on page 6 of the Office Action, has relied improperly upon remarks presented in support of the enabling disclosure of the application to justify the art-based rejection. The relationship between TRIMID-aminodextran and T-BSA is not a proper teaching or suggestion of the reference combination.

8

Chai-Gao et al. '802 does not supply what is missing from these references. As pointed out above, applicants' invention does not involve merely attaching TRIMID to aminodextran rather than to bovine serum albumin.

Lastly, it was well known from previous published experimental work that carbenes bind distinctly to all individual amino acids that may be present at the surface of a protein. That work is reported in the Journal of Photochemistry and Photobiology, B: Biology, 7, (1990) 277 to 287 entitled "Philicity of Amino Acid Side-Chains for Photogenerated Carbenes"; a copy of this article is provided also. A reading thereof shows that a carbene philicity scale was derived from the experiments reported therein and that carbene insertion (which leads to covalent bond formation) correlates with the polarity of the amino acid side chain and with the number of chemical bonds present in the side chain of the amino acid in question. Hydrophobic amino side chains are more carbene philic than hydrophilic amino acids and large amino acid side chains are also more carbene philic than small ones. The backbones of the dextran and aminodextran polymers consist of glucose monomers, meaning that the polymers are highly hydrophilic in

9

nature. The molecular surface exposes many small hydrophilic hydroxyl functions. Tightly bound water molecules adhere to dextran and its derivatives. Thus, a person of skill in the art would not expect the dextran surface to be carbene philic.

Contrary to the expectations discussed above, applicants discovered and have claimed that dextran is immobilized and that modified aminodextran itself could be used as a photo linker polymer. The rejection should be withdrawn.

The rejection of claims 3 and 6 to 10 under 35 USC 103 as unpatentable over Swan et al. '056 and Hubbell et al. '914 in view of Wessa et al. WO '631 is also respectfully traversed.

The arguments in support of patentability over Swan et al. '056 and Hubbell et al. '914 appeared elsewhere and previously in this paper. Wessa et al. WO '631, as Chai-Gao et al. '802, does not teach or suggest the invention as claimed. Applicants again direct the Examiner to the additional arguments here in support of the claims. Moreover, comparing the language of, e.g., instant claim 3 with the reference Derwent English language abstract (see especially the discussion of the process) reveals a disconnect that does not make the rejection a proper one.
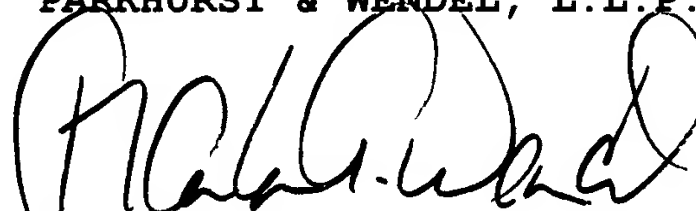
10

In view of the foregoing revisions and remarks it is respectfully submitted that the claims patentably define over the cited art and a USPTO paper to those ends is earnestly solicited.

The Examiner is requested to telephone the undersigned should additional changes be required in the case prior to formal allowance.

Respectfully submitted,

PARKHURST & WENDEL, L.L.P.

February 17,2005

Date

Charles A. Wendel
Registration No. 24,453

CAW/ch
Enclosures:
    Surface Immobilization of Biomolecules
       by light
    Philicity of Amino Acid Side-Chains for
       Photogenerated Carbenes

Attorney Docket No.: CSEM:065
PARKHURST & WENDEL, L.L.P.
1421 Prince Street
Suite 210
Alexandria, Virginia 22314-2805
Telephone: (703) 739-0220

PLEASE ACCEPT THIS AS
AUTHORIZATION TO DEBIT
OR CREDIT FEES TO
DEP. ACCT. 16-0331
PARKHURST & WENDEL

# PHILICITY OF AMINO ACID SIDE-CHAINS FOR PHOTOGENERATED CARBENES

HANS SIGRIST[†], MARC MÜHLEMANN and MAX DOLDER

*Institute of Biochemistry, University of Berne, Freiestrasse 3, CH-3012 Berne (Switzerland)*

## Summary

The selectivity of a diazirine-photogenerated carbene towards amino acid side-chains was investigated by analysing amino acid retention following photocoupling with an immobilized carbene precursor. The heterobifunctional photocross-linker 3-(trifluoromethyl)-3-(*m*-isothiocyanophenyl)diazirine was synthesized and coupled to fibre glass. Photoinduced amino acid binding to the solid support was analysed. The immobilized diazirine-photogenerated carbene preferentially binds to cysteine and aromatic amino acids. Amino acids carrying sulphur or oxygen as side-chain heteroatoms are, in general, more carbene-philic than amino acids with aliphatic side-chains. Marginal carbene insertion is obtained with glycine. On the basis of the empirically determined photocoupling capacities of the applied amino acids, a carbene philicity scale has been established. For homologous amino acids, carbene selectivity partly correlates with their hydrophobicity and the number of chemical bonds. Consequences of this distinct binding capacity are discussed with respect to photoselective protein modification.

## 1. Introduction

Photogenerated carbenes are exceptionally reactive and can be used for the timed initiation of covalent bond formation [1, 2]. Carbene insertion reactions into available chemical bonds occur within micro-seconds [3]. In biochemical studies, a wide selection of hydrophobic, diazirine-derived carbene precursors have been used for chemical exploration of the hydrophobic membrane core [4–9]. Hydrophobic labelling studies have led to the identification of transmembrane protein segments and have contributed to the

---

[†]Author to whom correspondence should be addressed.

experimental substantiation of the amphipathic helix concept [10]. Heterobifunctional reagents which provide a photoactivable carbene precursor are preferred probes for site-directed cross-linking. The reagents are applied to native, folded proteins [11, 12] or are introduced pretranslationally in the form of charged tRNAs which carry carbene-generating amino acid analogues [13, 14].

Documentation of carbene reactivity with individual amino acid side-chains is fragmentary. To date, systematic investigations have not been carried out. It is generally assumed that carbenes are capable of reacting with most, if not all of the amino acid side-chains, including aliphatic residues. Preferred labelling of sulphur-containing amino acids (cysteine, methionine) and aromatic amino acids has been experimentally documented [5, 7, 8, 10, 15]. The reactivity of photogenerated carbenes with amino acid side-chains is ambiguous. However, cognizance of distinct carbene-binding preferences is a prerequisite for thorough interpretation of carbene-derived protein modifications.

In this study the reactivity of a diazirine-derived, photogenerated carbene with amino acids has been investigated in a solvent-free solid-phase system. The carbene precursor 3-(trifluoromethyl)-3-(m-isothiocyanophenyl)diazirine (TRIMID) is covalently bound by thiocarbamoylation to a glass fibre support. Applied amino acids are then photocoupled and the photoinduced amino acid retention on washed supports is quantified.

## 2. Experimental procedures

### 2.1. Materials

Radiolabelled amino acids ($[^{14}C]$ or $[^{35}S]$) were purchased from Amersham and stored in 2% (v/v) ethanol at $-20$ °C (U-$^{14}$C-labelled L-Ala, L-Asn, L-Asp, L-Gln, L-Glu, L-Gly, L-His, L-Ile, L-Leu, L-Phe, L-Pro, L-Ser, L-Thr, L-Tyr, L-Val, L-[methyl-$^{14}$C]-Met, L-[methylene-$^{14}$C]-Trp). L-[U-$^{14}$C]-Lys, L-[U-$^{14}$C]-Arg and L-[$^{35}$S]-Cys were obtained as monohydrochlorides. (Ala, alanine; Arg, arginine; Asn, asparagine; Asp, aspartic acid; Cys, cysteine; Glu, glutamic acid; Gln, glutamine; Gly, glycine; His, histidine; Ile, isoleucine; Leu, leucine; Lys, lysine; Met, methionine; Phe, phenylalanine; Pro, proline; Ser, serine; Thr, threonine; Trp, tryptophan; Tyr, tyrosine; Val, valine.) Thiophosgene (purum) was purchased from Fluka and used without further purification. Whatman GF/C glass filter paper was used. Reagents used for the derivatization of glass filters were of sequenal grade, purchased either from Fluka, Merck or Pierce Chemicals. The following instruments were used for the chemical characterization of TRIMID: Uvicon 810 spectrophotometer; Perkin–Elmer 782 IR spectrophotomer; Varian XL-100 $^1$H nuclear magnetic resonance (NMR) spectrometer; CH 7A Varian MAT mass spectrometer.

## 2.2. Methods

### 2.2.1. Synthesis of 3-(trifluoromethyl)-3-(m-isothiocyanophenyl)-diazirine (TRIMID)

The heterobifunctional photoreagent TRIMID was synthesized by reacting 3-(trifluoromethyl)-3-(m-aminophenyl)diazirine with thiophosgene. The parent substance 3-(trifluoromethyl)-3-[m-(formylamino)phenyl]diazirine was prepared with minor modifications according to ref. 16. The formylated diazirine (120 mg, 0.52 mmol) was deformylated at ambient temperature with a mixture of 3 ml methanol and 1 ml HCl (37% by volume). NaOH (6 M, 3 ml) was added to the cooled (4 °C) solution and the arylamine was extracted with chloroform (4 ml). $NaHCO_3$ (5% w/v in $H_2O$, 650 $\mu$l) was then added to the chloroform extract. The suspension was stirred and cooled to 0 °C. The reaction was initiated by the addition of thiophosgene (133 $\mu$l) and product formation was controlled by thin layer chromatography (TLC). The stirred mixture was left at 0 °C for 20 min and for an additional 10 min at ambient temperature. The reaction mixture was washed with 1 M NaCl and $H_2O$. Excess solvent and thiophosgene were removed by a stream of nitrogen. The dried product was dissolved in chloroform and purified by preparative TLC on silica gel or flash chromatography. With this procedure 51 mg TRIMID (0.21 mmol) was obtained. Yields varied depending on the quality (age) of the thiophosgene and the quantities of educt used.

### 2.2.2. Chemical characterization of TRIMID

TLC (petrolether–ether, 1:1 (v/v)) $R_f$ 0.63; TLC (petrolether–ethyl acetate, 30:1 (v/v)) $R_f$ 0.52. Spectral absorbance: TRIMID has a significant diazirine absorption band with a maximum at 348 nm ($\epsilon_{348} = 446$ $M^{-1}$ $cm^{-1}$ in chloroform). Intense IR bands are recovered at 2080 $cm^{-1}$ (NCS), 1605 $cm^{-1}$ (N=N) and 1160 $cm^{-1}$ (C–F). $^1$H NMR spectra (in $CDCl_3$) confirm the meta substitution of the benzene ring: $\delta$ 7.02 (S, 1H), $\delta$ 7.1 (D, 1H), $\delta$ 7.28 (D, 1H) and $\delta$ 7.4 (T, 1H). Mass spectrometry indicates a molecular weight of 243 and reveals an M $-$ 28 fragment at 215 m/e formed on release of $N_2$.

### 2.2.3. Preparation of photolabel-derived glass filters

GF/C glass fibre sheets were aminopropylated according to ref. 17, yielding 12.4 nmol $NH_2$ groups per milligram of glass fibre. Procedures described by Sarin et al. [18] were followed for $NH_2$ group quantitation. Dried aminopropylated glass sheets were cut into discs of 0.8 cm in diameter and incubated with a tenfold molar excess of TRIMID in chloroform–triethylamine (200:0.1 (v/v)). The reaction was carried out for 20 min at 50 °C on a labshaker. Photolabel-derived glass filters were then washed individually with chloroform (16 ml) followed by 4 mM Cys (4 ml) and chloroform (6 ml). Aqueous Cys was used to remove unreacted TRIMID. By dithiocarbamoylation the reagent becomes soluble in aqueous media. The extent of TRIMID binding was 1.8 nmol photolabel per milligram of glass filter. The thiocarbamoylation reaction was quantitated with 3-(trifluorome-

thyl)-3-{p-[$^{125}$I]-iodo-m-isothiocyanophenyl}diazirine, a radiolabelled analogue of TRIMID.

### 2.2.4. Photoactivation

Carbene precursors were photoactivated with an HBO 350 mercury lamp (Osram). The power supply (SVX 1530) was obtained from Mueller Electronics, F.R.G. Photolabel-derived fibre glass discs were placed between two quartz slides in a custom-made holder. The holder was positioned 45 cm from the light source. Samples were photoactivated (controlled lamp power, 200 W) with filtered light (Schott WG 320 and a 1 cm layer of saturated $CuSO_4$ in $H_2O$; transmission band width, 320–550 nm) for a controlled length of time (Timer MU4, Mueller Electronics). Fibre glass discs were flushed with argon during photoactivation.

### 2.2.5. Photocoupling of amino acids

TRIMID-derived glass fibre discs were soaked with 20 μl ethanol (ethanol containing 0.1% (v/v) ammonia (25%) for Lys HCl and Arg HCl) before radiolabelled amino acids (0.66 nmol, 15 μl) were applied. Acidic solutions of Cys HCl were used to avoid disulphide formation. Loaded discs were mounted into the disc holding device and the disc chamber was flushed with argon 2 min before and during photoactivation. Samples were irradiated for 12 min, 6 min for each filter face. Control samples were prepared by applying identical quantities of radioactive amino acids to TRIMID-derived glass filter discs without light activation. Photoactivated and control discs were then thoroughly washed with solvents, applied in the following sequence and quantities: 1 M NaCl (20 ml), $H_2O$ (10 ml), ethanol (10 ml), chloroform (10 ml), toluene (10 ml). Washed discs were analysed for radioactivity by liquid scintillation counting. The amount of photocoupled amino acid was determined on the basis of the specific radioactivity. The extent of photoinduced (net) amino acid retention was obtained by subtracting the amount of unspecifically absorbed radiolabel (control) from the radioactivity recovered in photoactivated filter discs. Relative carbene philicity values are defined as the fraction of total net amino acid binding. The sum of net binding for all amino acids is set to 100%.

## 3. Results and discussion

### 3.1. Amino acid side-chains are distinctly carbene-philic

Reaction conditions described for aminopropylated glass modification with phenylene-diisothiocyanate [19] were used to successfully bind the newly described photocross-linker on glass supports. The product is a photolabel-derived glass fibre (Fig. 1) which, in this study, was used to rate the reactivity of the immobilized photogenerated carbene with free amino acids. The photoreaction was carried out in a chemically inert environment (argon atmosphere) where the solvent concentration was marginal. Photocoupling
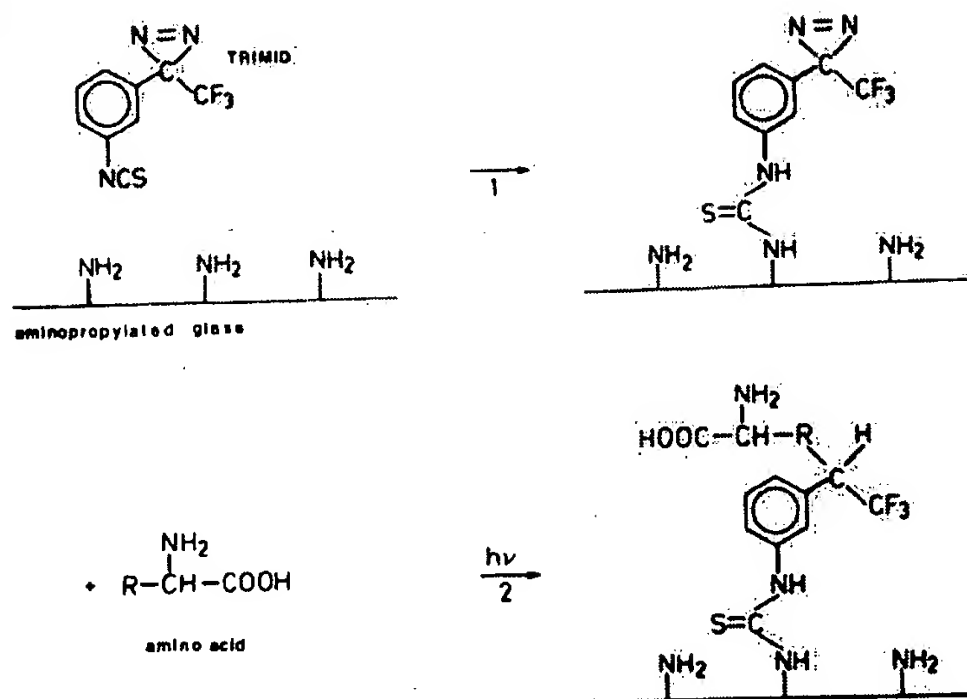
Fig. 1. Photoimmobilization of amino acids on glass fibre: step 1, photolabel binding to aminopropylated glass; step 2, photocoupling of amino acids.

of amino acids was thus attained in a system where the mobility of both reactants was restricted. The experimental conditions described allow the immobilization of those amino acids which reside within linking distance during the lifetime of the photogenerated carbene. In accordance with published diazirine labelling studies [5, 7–10, 15], photoactivation of either filter face was terminated after 6 min. Non-specific adsorption was observed for all amino acids. Within the set of amino acids tested, values for light-independent (unspecific) amino acid retention varied from 5% to 20% of the light-activated samples, although desorption of non-covalently bound amino acids was carried out. Desorption requires extensive washing of the filter discs with various solvents counteracting ionic (1 M NaCl), hydrophobic (ethanol, chloroform) and $\pi$–$\pi$ (toluene) interactions. Relative carbene philicities were derived from the averaged yield of covalent photoinduced amino acid binding. The results are shown in Fig. 2 and the numerical values are summarized in Table 1.

Since all amino acids except Pro have the common structure $NH_2$–HCR–COOH, photoinduced (net) amino acid retention exceeding the Gly value is implicitly side-chain specific. The binding studies indicate that aromatic side-chains including His imidazole, are favoured for carbene addition and insertion reactions (Trp > Tyr, His > Phe). Sulphur-containing amino acids (Cys, Met) and amino acid side-chains with oxygen heteroatoms are favourably photocoupled. Lowest binding yields are obtained for Gly (R = H). Considering the extremes, it is notable that carbene philicities of Cys and Gly differ by a factor of 45. With reference to studies on carbene selectivity in organic reactions, it has been shown that dicarbomethoxy carbenes react approximately four times faster with the sulphur atom of dimethylsulphide than with the
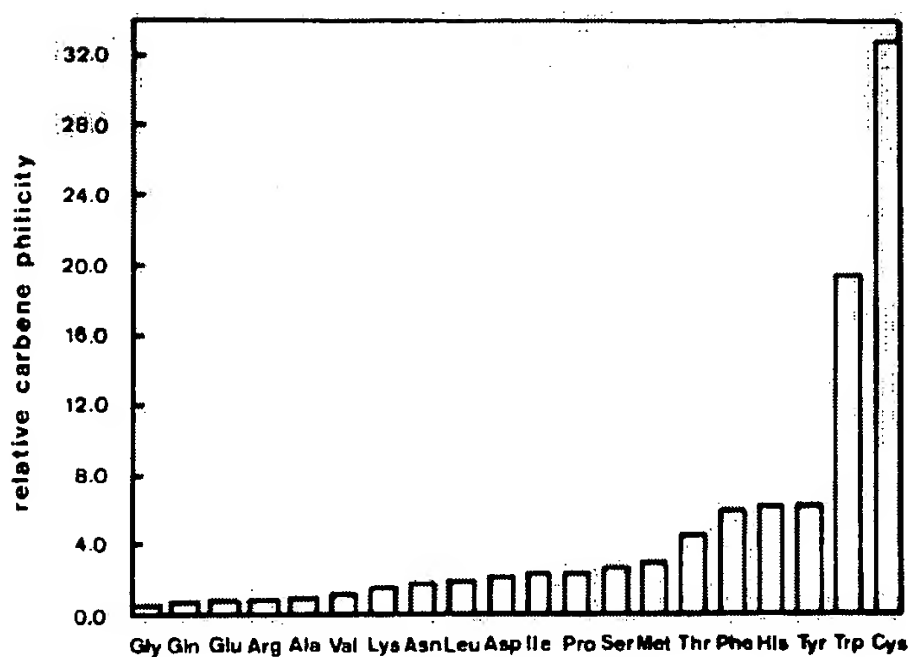
282



Fig. 2. Carbene philicity of amino acids. Radiolabelled amino acids were photocoupled to TRIMID-derived glass fibre discs and thoroughly washed with 1 M NaCl, $H_2O$, ethanol, chloroform and toluene. The extent of photoinduced (net) amino acid retention was analysed by liquid scintillation counting of the washed fibre discs. Averaged coupling yields were determined and relative carbene philicities were calculated. Individual carbene philicity values are summarized in Table 1.

cyclohexene double bond [20]. It has also been described that the insertion into the oxygen–hydrogen bond is ten times faster than the insertion into the primary carbon–hydrogen bond when reacting alcohols with diazomethane in the gas phase [21]. These examples confirm our observed trends in carbene selectivity, although they have limitations in that reactants and reaction conditions differ from those applied in this study.

### 3.2. The control of carbene insertion into amino acids is multifactorial

Several factors inherent to the individual amino acids have been correlated with the observed rate of photoselected coupling. Carbene philicity does not correlate with $pK_1$, $pK_2$ or the isoelectric points of the amino acids. This result indicates that there is no preference for carbene binding to the $NH_2-HCR-COOH$ structure, common to all amino acids. However, amino acid side-chains have distinct molecular structures and provide multiple sites for addition or insertion of the photogenerated carbene. Therefore structural and physicochemical characteristics of the side-chains may determine the extent of carbene binding.

The empirically derived carbene philicity parameters (Table 1) correlate in part with the hydrophobicity of amino acid side-chains. Figure 3 shows the correlation of carbene philicity with the $\pi$ factors of aliphatic amino acids; $\pi$ factors characterize the intrinsic side-chain hydrophobicity. They have been derived from the partition of $N$-acetyl-amino acid amides

TABLE 1

Carbene philicity values

| Amino acid | Net amino acid binding (pmol mg⁻¹ glass) | Relative carbene philicity (%) |
|---|---|---|
| Gly | 1.44 | 0.71±0.2 |
| Gln | 1.61 | 0.79±0.03 |
| Glu | 1.79 | 0.88±0.26 |
| Arg | 1.89 | 0.93±0.33 |
| Ala | 2.12 | 1.04±0.12 |
| Val | 2.60 | 1.28±0.71 |
| Lys | 3.38 | 1.66±0.02 |
| Asn | 3.77 | 1.85±0.10 |
| Leu | 4.17 | 2.05±0.25 |
| Asp | 4.52 | 2.22±0.31 |
| Ile | 4.89 | 2.40±0.12 |
| Pro | 4.97 | 2.44±0.09 |
| Ser | 5.56 | 2.73±0.13 |
| Met | 6.33 | 3.11±0.86 |
| Thr | 9.67 | 4.75±0.55 |
| Phe | 12.40 | 6.09±0.37 |
| His | 12.81 | 6.29±1.15 |
| Tyr | 13.04 | 6.40±0.42 |
| Trp | 39.67 | 19.47±2.48 |
| Cys | 67.05 | 32.91±2.74 |

Net amino acid binding and relative carbene philicity values are calculated from different sets of experiments each providing triplicate values for sample and control.
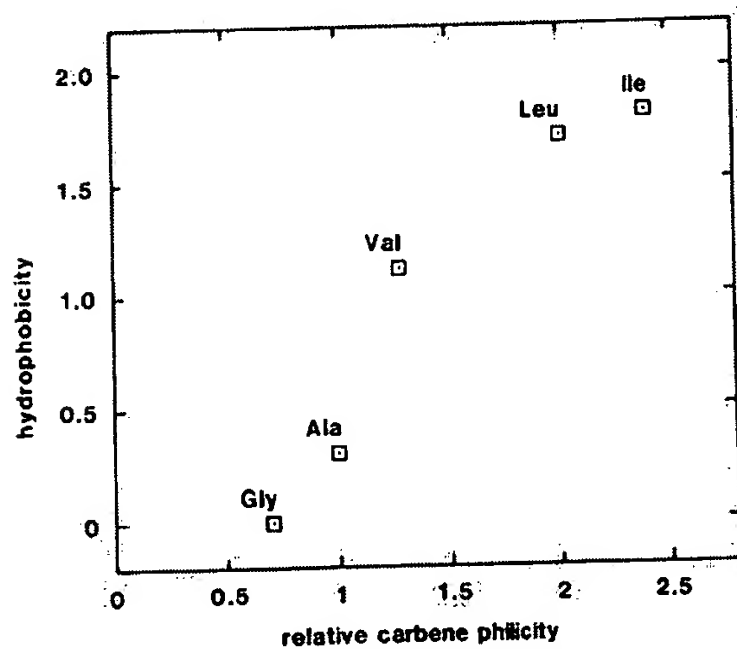
Fig. 3. Correlation of amino acid hydrophobicity ($\pi$ factors [22]) with experimentally derived carbene philicity values (Table 1).

$(CH_3-CO-NH-HCR-CONH_2)$ in octanol–water [22]. Aliphatic amino acids are increasingly carbene-philic with increasing hydrophobicity. A similar result is obtained by correlating amino acid binding selectivities with the hydrophobicity indices reported by Kyte and Doolittle [23].

Correlation of the number of chemical bonds per amino acid with the experimentally determined side-chain reactivity indicates that aliphatic amino acids follow the rule of C–H insertion (Fig. 4). Coupling yields decrease as the chain length decreases (Leu, Ile > Val > Ala > Gly).

In addition to the type and number of available bonds, the interaction between the generated carbene and individual amino acids is further determined by steric factors, the overall electronic structure of the reacting side-chain and the chemical properties of the photogenerated carbene (singlet vs. triplet carbene). Although, in theory, differential orbital energies of the reacting species could be calculated [24] for the prediction of binding selectivities, it would be most difficult to define steric parameters of amino acid side-chains in proteins and peptides.

### 3.3. Carbene selectivity and (membrane) protein modification

The empirically established carbene selectivities (this study) were compared with carbene labelling patterns published for several membrane proteins. Photoinduced modification of membrane-integrated parts of membrane proteins with diazirine-generated carbenes yields a predominant modification of sulphur-containing and aromatic amino acids (Table 2). A preference for Met labelling is obvious. It is further noted that the only Cys occurring in
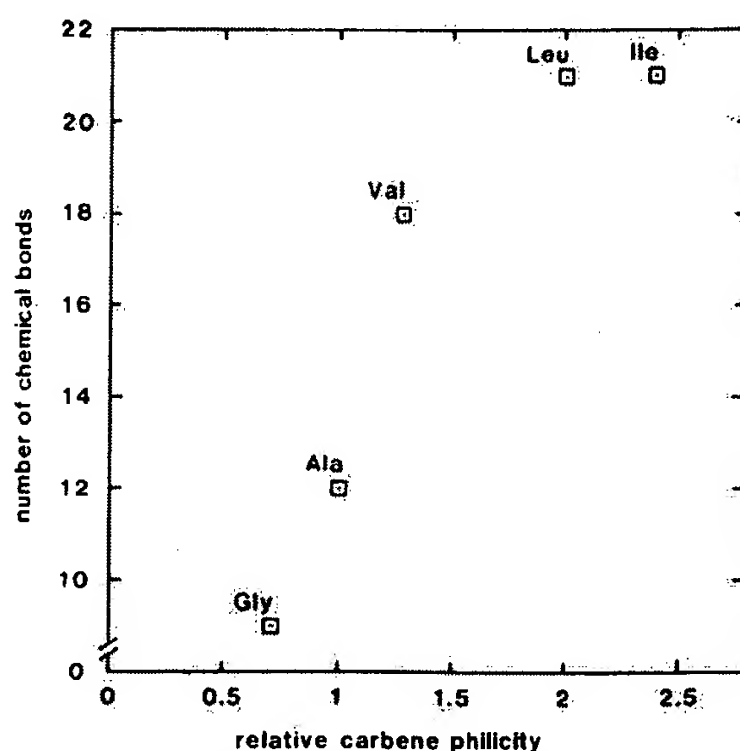


Fig. 4. Correlation of the number of chemical bonds per amino acid (aa) with relative carbene philicity.

TABLE 2

Carbene labelled amino acids in proteins

| Carbene label | Protein | Carbene labelled amino acids | Reference |
|---|---|---|---|
| TID | Proteolipid (Mito) | $Met_{73}$, $Met_{77}$, $Phe_{70}$, $Ser_{20}$, $Met_9$ | [7] |
| TID | Bacteriorhodopsin (CNBr 9a) | $Leu_{87}$, $Thr_{90}$, $Phe_{88}$, $Trp_{86}$, $Leu_{94}$ | [5] |
| TID | $F_1F_0$ATPase, b subunit | $Cys_{21}$, $Phe_{17}$, $Trp_{28}$, $Phe_{14}$, $Leu_8$ | [10] |
| TID | $F_1F_0$ATPase, c subunit | $Met_{11}$, $Met_{64}$, $Met_{75}$, $Phe_{76}$, $Leu_{19}$ | [10] |
| TID | Light-harv. protein B870-$\alpha$ | $Phe_{20}$, $Ile_4$, $Phe_8$, $Leu_7$, $Trp_5$ | [8] |
| Diazirine-PC | Light-harv. protein B870-$\alpha$ | $Trp_5$, $Ile_4$, $Phe_{20}$, $Phe_8$, $Leu_{28}$ | [8] |
| Diazirine-PC | PC-transfer protein | $Tyr_{176}$, $Met_{73}$, $Val_{171}$, $Asp_{177}$ | [15] |
| Diazirine-PC | Glycophorin | $Glu_{70}$ | [9] |

Soluble and membrane proteins modified *in situ* by diazirine-photogenerated carbenes are summarized in this table. Reagents applied are either the hydrophobic membrane label 3-(trifluoromethyl)-3-(m-[$^{125}$I]-iodophenyl)diazirine (TID) or phosphatidylcholine with a diazirine-derived acyl chain (diazirine-PC). Proteins are listed whose photolabel binding sites have been identified on the sequence level. From each peptide, the five most prominently labelled amino acids are cited in decreasing carbene binding order. They are identified by the sequence number if applicable.

the carbene labelled and sequenced polypeptides mentioned in Table 2 is significantly modified by the hydrophobic carbene. In general, *in situ* carbene labelled amino acids are, with few exceptions [9], identical with those characterized in this study as highly carbene-philic.

Due to the exceptionally low reactivity of Gly, it is expected that Gly-rich domains will barely indicate transmembrane disposition by 3-(trifluoromethyl)-3-(m-[$^{125}$I]-iodophenyl)diazirine (TID) labelling procedures. This has been observed on hydrophobic modification of the c subunit of *Escherichia coli* $F_1F_0$ ATP synthase [10]. The extent of carbene-induced modification seems to be determined by both the physical accessibility and the intrinsic reactivity of the amino acid side-chains. In general, knowledge of individual amino acid binding selectivities towards diazirine-photogenerated carbenes may contribute to an improved interpretation of protein modification data. Moreover, the established selectivity of carbene binding has practical consequences for judicious protein stabilization by intramolecular cross-linking and efficient protein immobilization on solid supports.

# References

1 V. Chowdhry and F. H. Westheimer, Photoaffinity labeling of biological systems, *Annu. Rev. Biochem.*, *48* (1979) 293–325.

2 R. A. G. Smith and J. R. Knowles, Aryldiazirines. Potential reagents for photolabeling of biological receptor sites, *J. Am. Chem. Soc.*, *95* (1973) 5072–5073.

3 H. Bayley, Photogenerated reagents in biochemistry and molecular biology, in T. S. Work and R. H. Burdon (eds.), *Laboratory Techniques in Biochemistry and Molecular Biology*, Elsevier, Amsterdam, 1983, pp. 8–61.

4 H. Bayley and J. R. Knowles, Photogenerated reagents for membranes: selective labeling of intrinsic membrane proteins in human erythrocyte membrane, *Biochemistry*, *19* (1980) 3883–3892.

5 J. Brunner, A. J. Franzusoff, B. Luescher, C. Zugliani and G. Semenza, Membrane protein topology: amino acid residues in a putative transmembrane $\alpha$-helix of bacteriorhodopsin labeled with the hydrophobic carbene-generating reagent 3-(trifluoromethyl)-3-($m$-[$^{125}$I]iodophenyl)diazirine, *Biochemistry*, *24* (1985) 5422–5430.

6 D. Pradhan and K. Lala, Photochemical labeling of membrane hydrophobic core of human erythrocytes using a new photoactivable reagent 2-[$^3$H]diazofluorene, *J. Biol. Chem.*, *262* (1987) 8242–8251.

7 J. Hoppe, D. Gatti, H. Weber and W. Sebald, Labeling of individual amino acid residues in the membrane-embedded $F_0$ part of the $F_1F_0$ ATP synthase from *Neurospora crassa*, *Eur. J. Biochem.*, *155* (1986) 265–272.

8 H. Meister, R. Bachofen, G. Semenza and J. Brunner, Membrane topology of light-harvesting protein B870-$\alpha$ of *Rhodospirillum rubrum* G-9$^+$, *J. Biol. Chem.*, *260* (1985) 16 326–16 331.

9 A. Ross, R. Rhadhakrishnan, R. J. Robson and H. G. Khorana, The transmembrane domain of glycophorin A as studied by crosslinking using photoactivatable phospholipids, *J. Biol. Chem.*, *257* (1982) 4152–4161.

10 J. Hoppe, J. Brunner and J. Jorgensen, Structure of the membrane-embedded $F_0$ part of $F_1F_0$ ATP synthase from *Escherichia coli* as inferred from labeling with 3-(trifluoromethyl)-3-($m$-[$^{125}$I]iodophenyl)diazirine, *Biochemistry*, *23* (1984) 5610–5616.

11 H. Sigrist, H. P. Michel and E. Kislig, Selective covalent modification of bacteriorhodopsin, in Y. A. Ovchinnikov (ed.), *Retinal Proteins*, VSP Science Press, Utrecht, 1987, pp. 295–306.

12 H. Ok, Ch. Caldwell, D. R. Schroeder, A. K. Singh and K. Nakanishi, Synthesis of optically active 3-diazoacetylretinals with triisopropylphenylsulfonylhydrazone, *Tetrahedron Lett.*, *29* (1988) 2275–2278.

13 M. Wiedmann, T. V. Kurzchalia, H. Bielka and T. A. Rapoport, Direct probing of the interaction between the signal sequence of nascent preprolactin and the signal recognition particle by specific cross-linking, *J. Cell Biol.*, *104* (1987) 201–208.

14 J. Brunner, G. Baldini and G. Semenza, Aminoacylation of tRNAs with L-4'-(3-(trifluoromethyl)-$^3$H-diazirin-3-yl)phenylalanine, *Experientia*, *44* (1988) A1.

15 J. Westerman, K. W. A. Wirz, T. Berkhout, L. M. M. van Deenen, R. Radhakrishnan and H. G. Khorana, Identification of the lipid-binding site of phosphatidylcholine-transfer protein with phosphatidylcholine analogs containing photoactivable carbene precursors, *Eur. J. Biochem.*, *132* (1983) 441–449.

16 J. Brunner and G. Semenza, Selective labeling of the hydrophobic core of membranes with 3-(trifluoromethyl)-3-$m$-[$^{125}$I]iodophenyl)diazirine, a carbene-generating reagent, *Biochemistry*, *20* (1981) 7174–7182.

17 R. H. Aebersold, D. B. Teplow, L. E. Hood and S. B. H. Kent, Electroblotting onto activated glass, *J. Biol. Chem.*, *261* (1986) 4229–4238.

18 V. K. Sarin, S. B. H. Kent, J. P. Tam and R. B. Merrifield, Quantitative monitoring of solid-phase peptide synthesis by the ninhydrin reaction, *Anal. Biochem.*, *177* (1981) 147–157.

19 W. Machleidt and E. Wachter, New supports in solid-phase sequencing, *Methods Enzymol.*, *47* (1977) 263–277.

20 W. Ando, T. Yagihara, S. Tozune and T. Migita, Formation of stable sulfonium ylides via photodecomposition of diazocarbonyl components in dimethylsulfide, *J. Am. Chem. Soc.*, *91* (1969) 2786–2787.

21 J. A. Kerr, B. V. O'Grady and A. F. Trotman-Dickenson, The reaction of methylene with hydroxyl groups, *J. Chem. Soc., A*, (1967) 897–898.

22 J. L. Fauchere and V. Pliska, Hydrophobic parameters $\pi$ of amino-acid side chains from the partitioning of *N*-acetyl-amino-acid amides, *Eur. J. Med. Chem.*, *18* (1983) 369–375.

23 J. Kyte and R. F. Doolittle, A simple method for displaying the hydropathic character of a protein, *J. Mol. Biol.*, *157* (1982) 105–132.

24 R. A. Moss, Carbenic selectivity in cyclopropanation reactions, *Acc. Chem. Res.*, *13* (1980) 58–64.

## Note added in proof

TID labelling of the influenza hemagglutinin "fusion peptide" revealed $Phe_3$, $Ile_6$, $Phe_9$, $Trp_{14}$, $Met_{17}$ and $Trp_{21}$ as predominant sites for [$^{125}$I] TID binding. (C. Harter, P. James, T. Baechi, G. Semenza and J. Brunner, Hydrophobic binding of the ectodomain of influenza hemagglutinin to membranes occurs through the "fusion peptide", *J. Biol. Chem.*, *264* (1989) 6459–6464.) It is to be noted that the radioactivity recovered along the sequence of the [$^{125}$I] TID labelled "fusion peptide" largely coincides with the relative carbene philicity of the individual amino acids.

# Surface immobilization of biomolecules by light

Hans Sigrist
André Collioud
Jean-François Clémence
Hui Gao
Reto Luginbühl
Michael Sänger
Gajendran Sundarababu
University of Bern
Institute of Biochemistry
Freiestrasse 3
CH-3012 Bern, Switzerland

**Abstract.** Biomolecules performing specific biological functions on material surfaces are progressively employed in the development of miniaturized bioassays, biosensors, bioelectronic devices, and medical equipment. Device performance is improved with covalently immobilized bioconstituents. The unique advantages of using light-controlled reactions to achieve biomolecule immobilization on surfaces are addressed. On activation of introduced light-sensitive reagents, biomolecules are covalently linked to material surfaces. Procedures leading to light-dependent engineering of surfaces are exceptionally facile. Immobilization by light is compatible with biological functions, enabling surface patterning and molecular coating of materials. Current strategies and protocols are illustrated with selected examples of biomolecule photoimmobilization.

Subject terms: biomolecules; photoimmobilization; photopatterning; biomaterials; surface crosslinking; surface grafting.

Optical Engineering 34(8), 2339–2348 (August 1995).

## 1 Introduction

Biomolecules are made by nature for optimal function in living systems. They are essential parts of highly regulated multicomponent networks in cells, tissues, and organisms. Recently, biomimetic technology has initiated attempts to integrate biomolecules and biomolecule-based reactions into technical systems and devices.[1-4] Biodevices have gained increasing attention because of the versatile and the highly specific binding capability and functions of proteins, nucleic acids, complex carbohydrates, and even cells. The expression of biomolecule functions depends vitally on their folding and intrinsic physicochemical properties. Although many biomolecules retain their natural function *in vitro* even after isolation and removal from their balanced biological environments, there are fundamental adaptations required to achieve biomolecule integration into biomimetic constructs. Expression of bioselectivities and biospecificities on surfaces (*in tecto*) demands system engineering toward structural stability and biomolecule surface immobilization including molecular orientation and macromolecule self-assembly.

It is a challenging task to make biomolecules exhibit and retain their unique biological activities on the surface of nonbiological materials. The interactions between surfaces and biomolecules are governed by various types of forces occurring at biomolecule-substrate interfaces. Physical contacts within chemical bond distances ultimately lead to biomolecule immobilization, which is either physical adsorption (physisorption), receptor-mediated adsorption (bioaffinity), or covalent binding. The former two processes are reversible, the latter leads to the formation of a chemical bond and is irreversible.

Covalent and affinity-based immobilizations have been explored since the early stages of affinity chromatography. Established immobilization procedures have found practical application in enzyme technology and analytical diagnostics.[5] Applications mentioned aimed at the retention of bioselectivity, the specific activity being of secondary importance. Biomolecule-mediated recognition and repetitive analyte binding on micro- to nanometer sized surfaces, however, may ultimately depend on few sensing molecules. Unrestricted biological activity of the sensing biomolecules and uniform molecular orientation are considered mandatory for miniature biodevice function. Covalent binding of functionally active biomolecules to material surfaces has yielded first generation biointerfaces that became essential components of sensor devices, bioelectronic elements, diagnostic systems, and medical equipment.[3,6]

Immobilization of biomolecules by light offers the prospect of facile and addressable modulation of substrates and enables the fabrication of new materials with retained biological functions. This article focuses on practiced experimental approaches for light-dependent biomolecule immo-

bilization on surfaces. Aspects of photocrosslinking in solution and in biological systems are not covered. The unique advantages of light-controlled reactions on surfaces are addressed and selected examples of current strategies and protocols are presented.

## 2  Photogenerated Nitrenes, Carbenes, and Ketyl Radicals Form Covalent Links with Target Molecules and Materials

Light-dependent biomolecule immobilization demands the presence of mediating photosensitive reagents. Linked to biomolecules, synthetic polymers, or material surfaces, these reagents are generally activated by incident light of appropriate wavelength. Presently, a considerable number of photosensitive reagents are available, many of them can be purchased from commercial sources. Although most reagents have been characterized with respect to their ability to form inter- and intramolecular crosslinks,[7-9] some are currently used for biomolecule immobilization on different substrates.[10-12] Preferred photoreagents for immobilization on material surfaces are substituted arylazides, trifluoromethyl-aryldiazirines, and benzophenones. The chemical structures of these reagents and their light-induced reactions are outlined in Fig. 1. When activated with light, the reagents shown undergo distinct chemical processes that finally lead to the formation of covalent bonds between the photogenerated intermediates and target biomolecules or material surfaces.

### 2.1  Carbene Intermediates Photogenerated from Trifluoromethyl-Aryldiazirines

Diazirines absorb light of wavelength 350 nm. They have low extinction coefficients and can be handled under normal laboratory conditions (there are no precautions necessary to avoid exposure to light). Highly reactive carbenes are formed on photoactivation of trifuoromethyl-aryldiazirines. With trifluoromethyl-aryldiazirines, unintended dark reactions of generated intermediates are minimized.[8] Carbenes preferably insert into C–H, C–C, C=C, N–H, O–H, or S–H bonds. Photogenerated carbenes are short-living species with microsecond half-lifetimes. They form covalent bonds with target substrates or molecules that are within molecular vicinity at the time of carbene generation. If target molecules are not present during the carbene lifetime, the intermediate will react with every molecular species present including water. The efficiency of carbene-mediated target molecule immobilization is therefore expectedly low unless biomolecule/surface contacts are formed prior to light activation. The formation of contacts may be promoted by substantial solvent removal before light exposure.[13] Solvent removal is, however, not mandatory.[14] With regard to light-dependent protein immobilization, the reactivity of diazirine-derived carbenes with amino acids has been explored. The reactivity of carbenes has been investigated by analyzing radiolabeled amino acid retention after photocoupling to thermochemically linked     3-(trifluoromethyl)-3-(m-isothiocyanophenyl) diazirine[15] on aminoalkylated glass fibers. Based on the empirically determined amino acid photoimmobilization, a carbenephilicity scale has been established. Relative carbenephilicities were derived from the average yield of covalent, light-dependent amino acid binding.[16] It was inferred from these studies that carbenes prefer heteroatom containing and
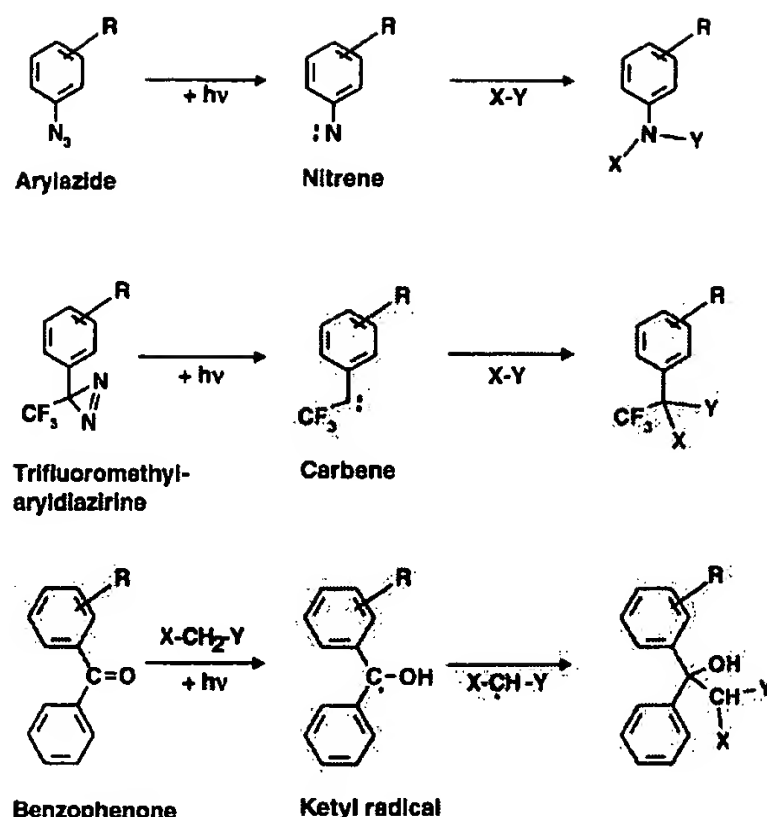


Fig. 1 Chemical structures and light-induced reactions of photoactivatable reagents.

aromatic amino acids. With respect to light-dependent protein immobilization, the results support the view that the amino acid composition in a surface domain of a protein may determine the preference of reaction with photogenerated carbenes.

### 2.2  Nitrene Intermediates Photogenerated from Arylazides

Arylazides generate highly reactive nitrene intermediates on photolysis. Nitrenes are isoelectronic with carbenes and undergo a variety of reactions. Phenylazides require light activation around 250 nm, which overlaps with the absorption maxima of biomolecules. The use of nitrophenyl azides that absorb UV light of about 350 nm circumvents the problem of irradiation damage to biomolecules. Nitrene intermediates photogenerated from phenylazides undergo rapid intramolecular ring expansion reactions leading to azacyclo-heptatetraene intermediates. These are highly electrophilic species and react with nucleophilic groups at a rate much slower than the direct nitrene insertion reaction. The unwanted ring expansion reaction can be minimized by the use of perfluorophenylazides. Although phenyl and nitrophenylazides have been extensively utilized in photolabeling studies over several years, the importance of perfluorophenylazides has been realized only during the last decade. It has been clearly established now that fluorine substitution raises the energy barrier to the ring expansion reaction of perfluorophenylnitrene to the corresponding azacycloheptatetraene. In other words, the perfluoro substitution increases the coupling efficiency of nitrenes including their rapid insertion into unactivated C–H bonds.[8,17-19]

In analogy to the study delineating the carbenephilicity of individual amino acids, relative nitrenephilicity values have been determined. For this purpose,

p-azidophenylisothiocyanate was thermochemically coupled to aminopropylated glass fibers. Radiolabeled amino acids were applied to modified supports and photoimmobilized. After removal of adsorbed amino acids, the extent of light-dependent amino acid immobilization was analyzed.[20] Romero et al.[21] have carried out similar studies comparing the relative photolabeling of various radiolabeled amino acids by Sepharose-coupled 4-azido salicylic acid in aqueous solution. Both studies indicate distinct reactivity characteristics for photogenerated nitrenes (aromatic > small aliphatic > "charged" amino acids).

### 2.3 Ketyl Radicals Photogenerated from Benzophenones

Benzophenones are precursors for the photogeneration of ketone biradicals. On photolysis at 350 nm, benzophenone readily forms a triplet biradical with a very high quantum yield.[22] This biradical is a facile hydrogen abstracting species. Hydrogen abstraction from target molecules and substrate surfaces precedes covalent benzophenone-mediated binding. Ideally, the resulting ketyl radical undergoes radical coupling with a hydrogen-abstracted center, resulting in a covalent bond. The reactivity of light-activated benzophenones differs significantly from photogenerated nitrenes and carbenes, in that ketyl radicals do not react with water. Excited molecular species return to the ground state if radical coupling does not occur. In practice, benzophenone-mediated immobilization reactions can be carried out in aqueous media. Generally, with otherwise identical experimental conditions, benzophenones need longer irradiation times than trifluoromethyl-aryldiazirines to achieve comparable extents of target molecule immobilization.

## 3 Biomolecule Immobilization by Light Is Compatible with Biological Functions and Topically Addressable

Although covalent biomolecule immobilization on material surfaces has been accomplished by thermochemical reactions, photoimmobilization has gained increased attention because of the compatibility with biological functions and the topical addressability.

### 3.1 Light-Dependent Reactions are Compatible with Biomolecule Functions

Two aspects applying to the compatibility of immobilization procedures deserve consideration: first, does the energy required to activate the photoreagent interfere with biomolecules and, second, do the reaction conditions used for thermochemical coupling hamper the biological functions? Nitrophenylazides, trifluoromethyl-aryldiazirines, and benzophenones are generally activated by irradiation at wavelengths ≥ 350 nm for which most biomolecules are transparent. Harmful interactions with endogeneous chromophores (aromatic amino acids, purines, pyrimidines) are thus avoided. Biological functions such as ligand binding, enzymatic activities, immunocomplexation, and deoxyribonucleic acid (DNA) base-pairing have been used to document the retention of biomolecule activities. These statements are based on the following experimental observations: biotin binding to photoimmobilized streptavidin is not affected[14,23] by irradiation at wavelengths ≥ 320 nm, although the biotin-

binding pocket is a complex arrangement of several aromatic amino acids including tryptophan.[24] Moreover, preservation of catalytic activity has been documented with trypsin photoimmobilized on liposomes[14] and numerous enzymes were found active after surface immobilization by light (Table 1). Experiments dealing with the oriented immobilization of immunoreagents have revealed that the specific immunoactivity was two to four times higher with photoimmobilized F(ab') antibody fragments than with physically adsorbed molecules.[25] Diazirine-based immobilization of an oligonucleotide probe (irradiance of 0.7 mW/cm² at 350 nm) has shown that photoimmobilized DNA specifically recognized its complementary DNA strand.[26] In summary, the observations mentioned strongly suggest that the applied photoimmobilization procedures are compatible with biological functions.

Considering the reaction conditions required for light-dependent immobilization, note that, in principle, target biomolecules can be applied in media that confer with the structural stability of the biomolecule. Photoimmobilization does not require invasive agents (activating agents) except appropriately dosed energy in the form of light, whereas the reaction medium composition, pH, and temperature can be adapted to the needs of the biomolecule. This is in contrast to thermochemical immobilization reactions where coupling efficiencies depend on the reaction conditions. In this case, the reaction parameters are optimized for the chemical reaction. Some biomolecules or complex biological systems, however, may tolerate only limited variations of bulk conditions and medium composition. In consequence, conditions used for thermochemical immobilization may structurally weaken or even denature target molecules.

### 3.2 Addressability

Topical addressability is a genuine property of photoactivatable reagents. The possibility of generating patterned surfaces attains increasing significance in multiple domain analytical systems and in the construction of biomimetic devices.[27,28] The power of this approach has been demonstrated by Jacobs and Fodor.[28] The authors provide convincing evidence that high-density arrays of distinct biomolecules can be constructed by mask alignment and the use of photodeprotection chemistries in peptide and oligonucleotide synthesis. Photopatterning can be attained either by masking techniques or, potentially, by direct laser writing.[29] Restrictions to photopatterned designs are few, technically imposed by the availability of appropriate masks, the light sources, and laser beam dimensions. Light-addressed biomolecule immobilization enables one not only to topically select the area but also to control the extent of biomolecule immobilization by exposure time variation.[30] An example for light-addressed patterning is presented in Fig. 2. A TiO₂/SiO₂ layer has been coated with the heterobifunctional, thiol-selective crosslinker N-[m-[3-(trifluoromethyl)diazirin-3-yl]phenyl]-4-maleimidobutyramide (MAD). The coated surface was covered with a photomask and exposed to 350-nm activating light. Physically adsorbed reagent was removed by washing and the photoimmobilized reagent was further derivatized with radiolabeled [³⁵S]-cysteine. Binding of the radioactive amino acid to the maleimido function of the photoimmobilized crosslinker was then detected by autoradiography. Replacing the radioactive amino acid by any thiolated biomolecule affords topically selective immobilization. In

**Table 1** Light-dependent immobilization of biomolecules.

| Immobilized biomolecules | Immobilization method | Substrate | References |
|---|---|---|---|
| *Arylazides as photoreagents* | | | |
| Sulfonated glycolipid | Surface grafting type A | Aminoalkylated glass | 56,57 |
| Enzymes | Surface grafting type A | Aminopropylated glass, organic polymers | 11,58 |
| Enzymes | Surface grafting type A | Aminoalkylated glass,silicon chips | 59,60 |
| Enzymes | Surface grafting type B | Polystyrene | 52 |
| Carbohydrates, Proteins | Photolinker polymer | Organic polymer | 42,61-63 |
| Bacteria, viruses | Surface grafting type A | Aminopropylated glass | 17 |
| Antibodies | Photolinker dimer | Glass | 64 |
| *Aryldiazirines as photoreagents* | | | |
| Amino acids | Surface grafting type A | Aminoalkylated glass fibres | 16 |
| Peptides | Surface grafting type A | Aminoalkylated glass fibres | 13,65 |
| Peptides | Surface grafting type C | Hydroxylated FEP | 25,40 |
| Oligonucleotides | Photolinker polymer mediated | Polystyrene | 26 |
| Proteins | Surface grafting type B | Cellulose, Nylon | 49,50 |
| Proteins | Surface grafting type B | Mica | 51 |
| Enzymes, proteins | Photolinker polymer mediated | Glass, Polyvinylidene difluoride | 13,41 |
| Antibodies | Photolinker polymer mediated | Polystyrene | 30 |
| Antibodies | Surface grafting type C | (BSA precoated) Polystyrene | 25 |
| Antibody fragments | Surface grafting type A | Silicon chips | 37 |
| Antibodies | Photolinker polymer mediated | $TiO_2/SiO_2$ waveguides | 44 |
| Antibody fragments | Surface grafting type A | Photosomes | 14,46 |
| *Benzophenones as photoreagents* | | | |
| Peptides | Surface grafting type C | Glass, PVA | 40 |
| Protein | Photolinker polymer | Polystyrene | 18 |
| Protein | Photolinker polymer | Polyurethane, Polyethylene | 66 |
| Protein | Surface grafting type A | Aminoalkylated glass beads | 67 |
| Antibodies | Surface grafting type A | Glass, Silicon chips | 37,43 |
| Antibodies | Photolinkerpolymer mediated | Polycarbonate | 68 |

Detailed descriptions of the major methods applied to achieve biomolecule immobilization by arylazides, trifluoromethyl-aryldiazirines, or benzophenones as photoreagents are given in Sec. 4.2. If multiple photolabel derivatized biomolecules are directly immobilized on substrates, it is referred to as the "photolinker polymer" method. "Photolinker dimer" represents procedures in which a low-molecular-weight homobifunctional crosslinker is used for biomolecule immobilization. Abbreviations: BSA, bovine serum albumin; FEP, fluorinated ethylene propylene; and PVA, polyvinylalcohol.

addition, oriented immobilization is attained if the thiol function is singular and accessible at the biomolecule surface.[25]

## 4 Strategies and Protocols for Light-Dependent Biomolecule Immobilization on Surfaces

### 4.1 Significance of Biomolecule/Surface Interactions in Photoimmobilization

Covalent biomolecule immobilization is initiated by molecular interactions of the target molecules with material surfaces. The formation of interfacial contacts is governed by the physicochemical properties of both the material and the

biomolecule surface. Multifactorial physical interactions lead to adsorbent binding.[31] The biomolecule surface is heterogeneous with respect to surface charge and topology, domain polarity, molecular packing density, and chemical reactivity. The larger the target molecule, the more interphase contacts are simultaneously established. If the sum of these individual forces acting on target molecules exceeds the strength of intramolecular binding forces, the target molecule will unfold, eventually denature, and lose its biological activity. Biointerface technology thus operates at the borderline between necessary initial surface interaction and biomolecule denaturation. Therefore, keeping biomolecules active at the interface demands control over high-energy noncovalent in-
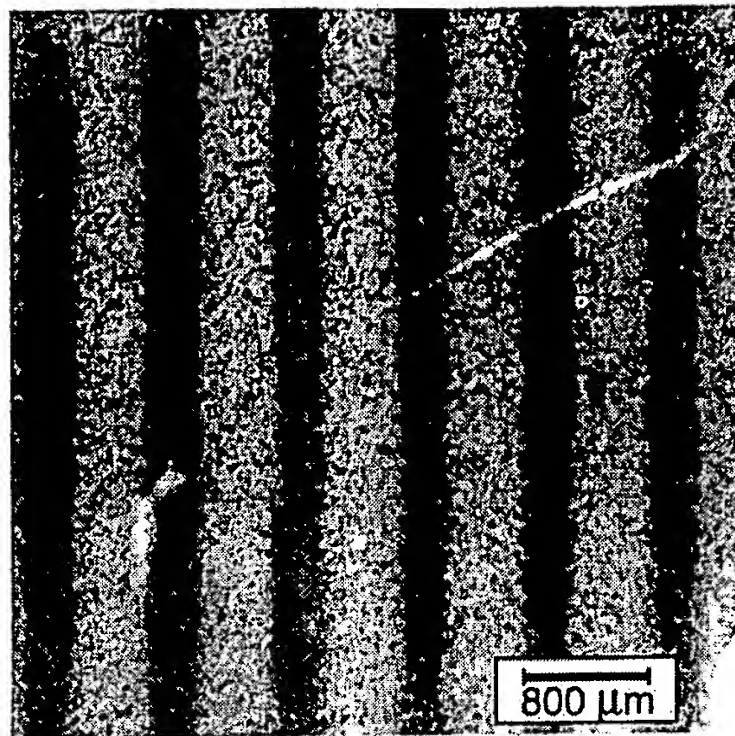
**Fig. 2** To demonstrate light dependency and covalency of interaction between $TiO_2/SiO_2$ and photogenerated carbenes, maleimido-trifluoroaryl-diazirine (MAD)-coated $TiO_2/SiO_2$ surfaces were exposed to activating light through a patterned mask. After light exposure, the substrate was incubated with [$^{35}$S]-cysteine. Excess radiolabel was washed off and the dried surface was exposed to an x-ray film. Dark areas (300 $\mu$m) correspond to surface grafted [$^{35}$S]-cysteine.

teractions. This goal can be attained considering either of the following precautions: Surfaces may be modified or functionalized to achieve desired surface characteristics and to suppress denaturing interface contacts between biomolecules and the support material.[32-34] Alternatively, the bulk environment of the biomolecule can be complemented with stabilizing agents (proteins, detergents) which reduce nonspecific adsorption[35,36] and help to conserve native structures during biomolecule adsorption and covalent binding.[30,37] Finally, the stability of the biomolecule itself can be enhanced by genetic engneering[38] or intramolecular crosslinking.[39]

### 4.2 Protocols for Light Dependent Biomolecule Immobilization

Photochemical linkages between material surfaces and biomolecules are formed by either surface grafting or photolinker polymer mediated procedures. *Surface grafting* entails thermochemical or photochemical functionalization of material surfaces with a heterobifunctional crosslinker (Fig. 3, grafting types A and B). Alternatively, the biomolecules to be immobilized are thermochemically derivatized with a photoreagent prior to light-dependent surface immobilization[40] (type C). General principles of surface grafting are exemplified in Fig. 3, the heterobifunctional reagent 3-(trifluoromethyl)-3-(m-isothiocyanophenyl) diazirine serving as linking reagent. *Photolinker-polymer-mediated immobilization* procedures require a polymer, which is substituted with photoactivatable groups along the polymer chain. The polymer may be of biological origin[41] or a synthetic copolymer.[42] For target biomolecule immobilization, the photolinker polymer is underlayered to, or mixed with, the biomolecules on the substrate and irradiated (Fig. 4). Light-dependent copoly-

merization leads to covalent immobilization of target (bio)molecules and simultaneous covalent binding of the polymer to material surfaces or other polymers. With either method, numerous types of biomolecules were photoimmobilized on materials differing in chemical composition and physical properties. Examples are summarized in Table 1.

### 4.3 Selected Examples of Light-Dependent Biomolecule Immobilization Strategies

In type A surface grafting methods, covalent surface derivatization of glass, $SiO_2$ or $TiO_2/SiO_2$ with terminally functionalized silanes has been used to design surfaces according to the needs of the biomolecule or biological system. Modification of hydroxylated surfaces with short-chain or long-chain polyfunctional silanes yields functionalized surfaces. Photoactivatable surfaces have been subsequently produced by thermochemical coupling of bifunctional photoreagents.[37,43] It has further been shown that the presence of serum albumin during target molecule physisorption retained the immunological activity of a monoclonal antibody.[30,37,44] In another study, Langmuir-Blodgett film deposition of long-chain amphiphilic nitrophenylazides on hydrophobic surfaces and subsequent photoimmobilization of bioactive proteins in monomolecular layers has been achieved.[45]

The formation of photoactivatable surfaces is not restricted to solid substrates. In a recent study, light-dependent immobilization of proteinaceous ligands to liposomes has been introduced as a rapid, noninvasive technique for the production of proteoliposomes.[14] The uniqueness of this method is the usage of $N'$-(1,2-dimyristoyl-sn-glycero-3-phosphoethyl)-$N$ -[$m$ -[3-(trifluoromethyl)diazirin-3-yl] phenyl]thiourea (PED), a bilayer-forming photoactivatable phospholipid analogue. Small unilamellar vesicles were formed from PED by ethanol injection. These vesicles are termed photosomes, emphasizing their light-dependent reactivity. On photoactivation of the PED head group diazirine, target proteins were covalently linked to the photosomes. Myoglobin, streptavidin, and trypsin served as reference proteins to elaborate the basic procedures for light-induced liposome functionalization. Photocoupling of myoglobin to photosomes yielded 15% liposome surface coverage. Coupling of streptavidin to photosomes provides a polyvalent system, which enables conjugation of any biotinylated ligand. Conjugates from $F(ab')_2$ and photosomes have been prepared, the efficiency of photoimmobilization being about 18% of applied $F(ab')_2$. Immobilized fragments were competent in forming immunocomplexes.[46]

*Type B surface grafting* is a most appreciated process to introduce selectively reactive functions on surfaces that do not possess suitable functional groups for thermochemical reactions (Fig. 3). Photoinduced polymerization of the crosslinking reagent is most probable if photolabels are adsorbed or spin coated and dried on the surface prior to photoactivation. The same concepts can be applied to *surface grafting type C*, which is appropriate for oriented biomolecule immobilization with terminally monofunctionalized biomolecules.

The following study may demonstrate how photoimmobilization technologies serve to pattern neuronal cells on material surfaces. Graf et al.[47] presented convincing evidence that the synthetic peptide CDPGYIGSR mediates cell adhesion and migration. The peptide binds specifically to a 67,000
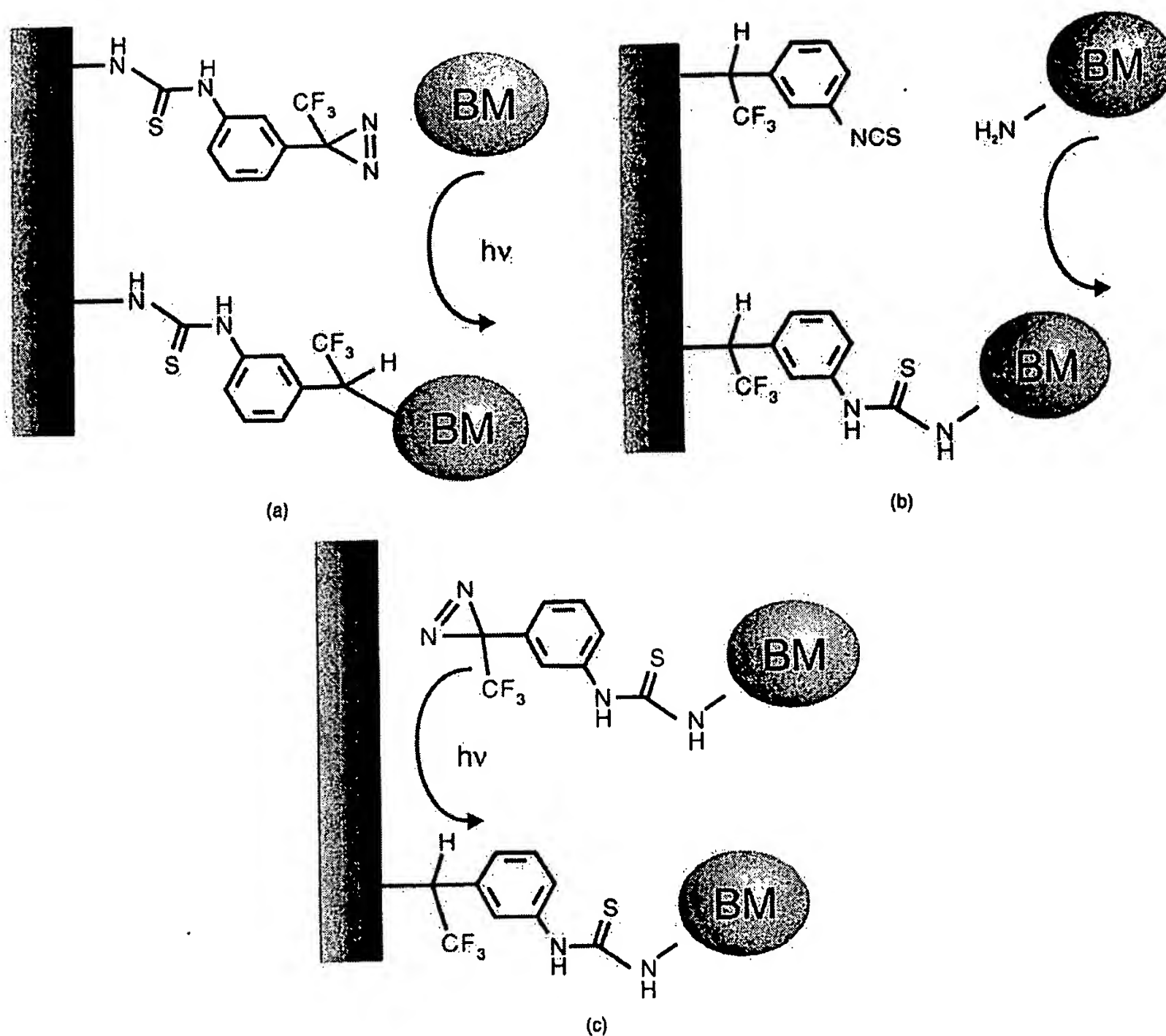
**Fig. 3** Biomolecule photoimmobilization on material surfaces by surface grafting procedures. Basic approaches are exemplified with the photocrosslinker 3-trifluoromethyl-3-(m-isothiocyanophenyl) diazirine. (a) Surface grafting type A: The material surface is modified to introduce a functional group suitable for thermochemical reaction with the bifunctional reagent. Biomolecules (BM) are then photochemically linked to the surface. (b) Surface grafting type B: A heterobifunctional crosslinking agent is photochemically linked to the material surface, yielding a thermochemically reactive substrate for biomolecule immobilization. (c) Surface grafting type C: The target biomolecule is site(mono)specifically modified with the crosslinker prior to photochemical coupling to the material surface.

Dalton nonintegrin cell surface receptor. In our studies, CDPGYIGSR has been terminally functionalized with the carbene-generating maleimido-trifluoromethyl-aryldiazirine, MAD. In conjunction with masking techniques, topically selective and oriented peptide binding onto hydroxylated fluorinated ethylene propylene has been achieved.[48] Neuronal cells attached selectively to the photoimmobilized peptide (Fig. 5). The specificity of the process has been documented by the fact that cell binding was suppressed in the presence of free peptide in solution. Preferred attachment and differentiation of cultured cells (neuroblastoma x glioma cell line NG 108-15) occurred in peptide modified domains.[40]

Several publications report on the photoimmobilization of peptides, proteins, structurally stable enzymes, and polyclonal antibodies on type B and C grafted surfaces.[25,40,49-52] Photolabeled nucleic acids can be prepared using azidederivatized parent nucleotides in oligonucleotide synthesis.[53] Alternatively, azido groups have been introduced into DNA by treatment with p-azidophenylglyoxal. This reagent reacts with terminal guanine residues to form an azido derivative that can then be photoactivated.[54] It is, however, factual that many biomolecules easily physisorb on grafted material surfaces. Nonspecific biomolecule binding can be suppressed by pretreatment of the surface with a protecting protein layer.
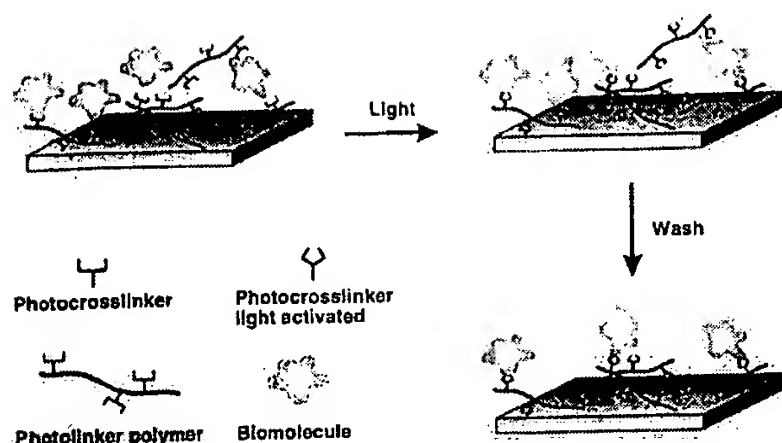
**Fig. 4** Photolinker-polymer-mediated immobilization of biomolecules.

Coating of polystyrene titer plates with bovine serum albumin, followed by photolabel immobilization and thermochemical immunoreagent binding, has yielded immunocompetent surfaces with suppressed nonspecific binding.[25]

Photolinker-polymer-mediated biomolecule immobilization of target peptides, carbohydrates, catalytically active enzymes, and antibodies with photolabel functionalized serum albumin or synthetic photolabeled copolymers is a rapid and convenient way to attach biomolecules to surfaces[13,42,55] (Fig. 4). The potential and limitations of photolabeled serum albumin mediated immobilization has been explored with immunoreagents. A sandwich-type assay system has been elaborated, which includes covalent photoimmobilization of

the first immunocomponent on polystyrene.[30] Fabrication of a regenerable optical immunosensor has been accomplished.[44] The essential information of the latter study is summarized in Fig. 6, which depicts an on-line record of the binding of an antigen/second antibody complex to a photoimmobilized antibody fragment on a $TiO_2/SiO_2$ waveguide layer.

## 5 Summary and Perspectives

Considering both the biological requirements and device engineering, there are definite advantages in using light for covalent biomolecule immobilization. Photoreagent-mediated reactions, which are initiated by irradiation at wavelengths $\geq 350$ nm, were found compatible with biological functions. Photoimmobilization processes are topically addressable. Moreover, light-dependent procedures make it possible to experimentally select the time of initiation and the extent of biomolecule binding, enabling multiple functionalization of surfaces. Light-mediated immobilization reactions are therefore considered important tools for the fabrication of biodevices. Extension of photopatterning technologies to submicro- or nanometer dimenions offers the prospect of single molecule immobilization. The successful covalent immobilization of various types of biomolecules illustrates the versatility and the potential of photochemical procedures. Moreover, covalently linked molecules may prove advantageous for obtaining high-quality images by high-resolution-scanning probe techniques and for the preparation of biomolecule functionalized tips.
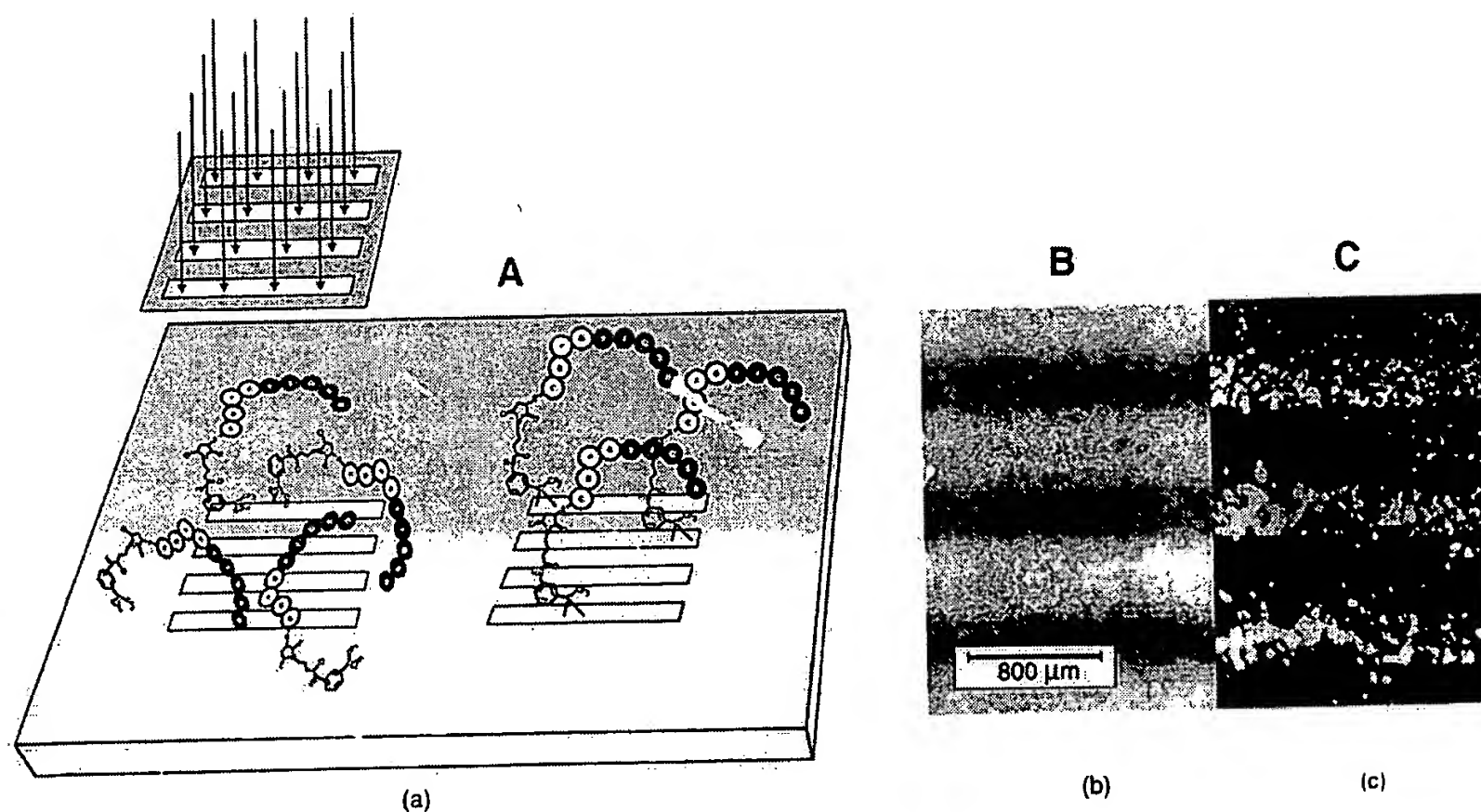


**Fig. 5** Photopatterning with the terminally photolabel functionalized oligopeptide, trifluoromethyl-diazirine maleimido-Cys-Asp-Pro-Gly-Tyr-Ile-Gly-Ser-Arg-NH₂ onto hydroxylated fluorinated ethylene propylene membranes (a). (b) A 300-μm photopattern of the (radioactive) peptide is visualized by autoradiography (scale bar, 800 μm). (c) Selective neuronal cell attachment on this substrate. The cells adhere almost exclusively on peptide modified areas with virtually no attachment to unmodified sections.
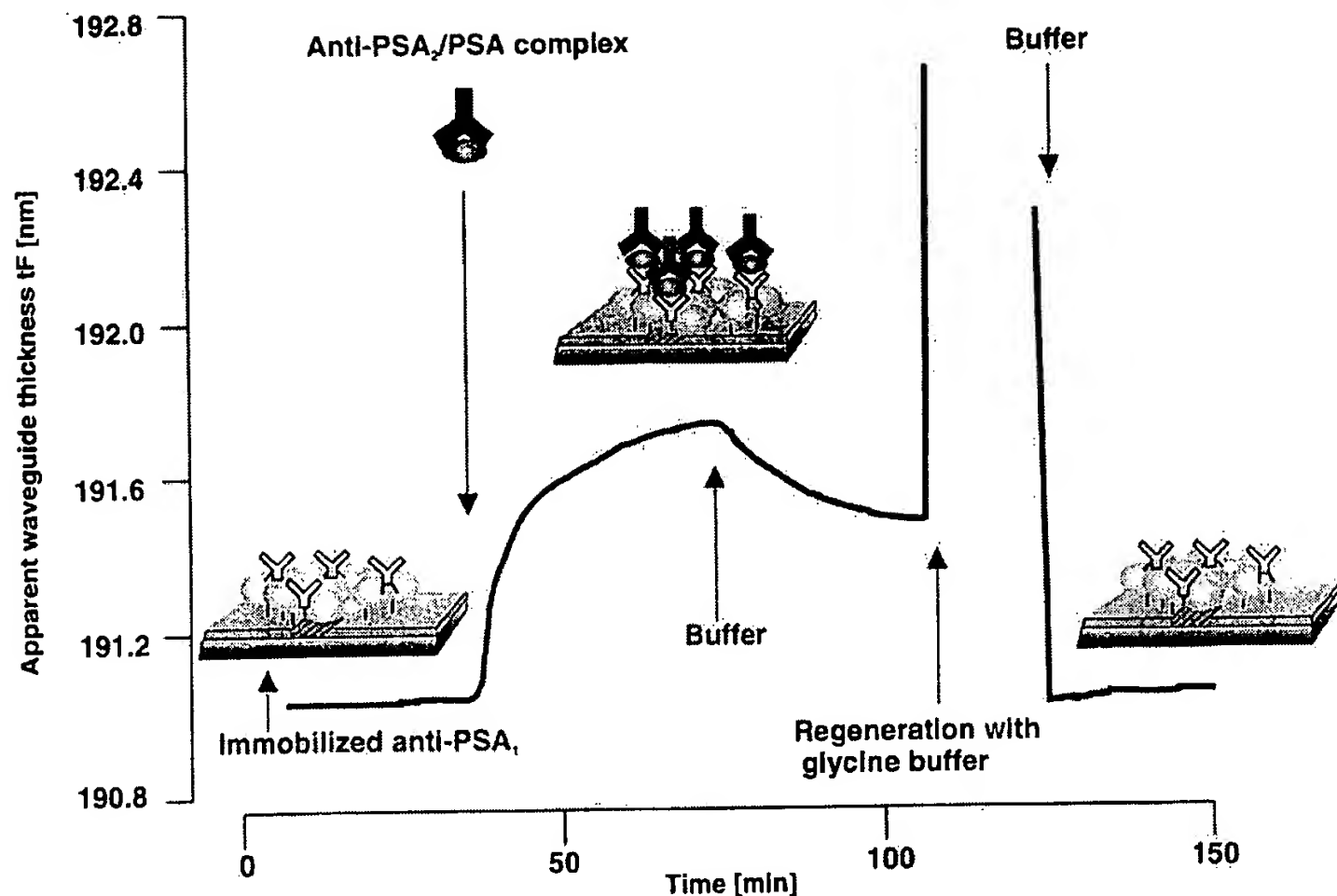
**Fig. 6** Reversible immunocomplex formation on a waveguide layer. Immunocompetent antibody $F(ab')_2$ fragments are photoimmobilized on a $TiO_2/SiO_2$ waveguide layer by photolinker-polymer-mediated procedures. The formation and dissociation of sandwichtype immunocomplexes is followed by recording the apparent waveguide thickness tF with a grating coupler sensor. $F(ab')_2$ fragments of a monoclonal, anti–prostate specific antigen (anti-$PSA_1$) antibody are photoimmobilized on the grating coupler sensor chip. Sandwichtype immunocomplexes are formed on addition of an antigen/second antibody immunocomplex (PSA/anti-$PSA_2$). Regeneration is attained by treating the surface with glycine buffer (pH 2.3). Subsequent washing with a pH 7.4 TRIS buffer restores an immunocompetent anti-$PSA_1$ sensor surface.[44]

Applied microsystem technology requires miniaturized devices to control and survey technical processes by input/output analysis. If analytes are liquids or gases, biospecific sensing is most appropriate because biomolecule-based detection systems provide exceptional specificity, molecular selectivity, and high efficiency. Biological systems are known to considerably use surfaces and compartmentalization to achieve efficient biological responses. In many instances, primary biological events (e.g., receptor activation by ligand binding) are potentiated in the course of signal propagation and avalanche effects are attained by multicomponent cooperativity. Progress in microstructuring of materials and facile immobilization of biomolecules set the basis for the construction of multicomponent biomimetic devices and semiconductor-based sensors. Furthermore, it is expected that the experimentally facile light-dependent immobilization procedures will facilitate the screening of factors that control cell attachment and differentiation. Light-induced patterning of bioactive recognition elements provides means for the fabrication of 2-D substrates in vitro and the prerequisites for future structing of 3-D biomimetic material systems.

### Acknowledgments

### References

1. U. B. Sleytr, P. Messner, D. Pum, and M. Sara, *Immobilised Macromolecules: Application Potentials*, Springer-Verlag, London (1993).
2. P. Connolly, "Bioelectronic interfacing: micro- and nanofabrication techniques for generating predetermined molecular arrays," *TIBTECH: Trends in Biotechnology* 12, 123–127 (1994).
3. M. Aizawa, "Molecular interfacing for protein molecular devices and neurodevices," *IEEE Eng. Med. Biol.* 13, 94–102 (1994).
4. B. D. Ratner, "New ideas in biomaterial science—a path to engineered biomaterials," *J. Biomed. Mat. Res.* 27, 837–850 (1993).
5. G. T. Hermanson, A. K. Mallia, and P. K. Smith, *Immobilized Affinity Ligand Techniques*, Academic Press, San Diego (1992).
6. F. T. Hong, *Molecular Electronics Biosensors and Biocomputers*, Plenum Press, New York and London (1989).
7. S. S. Wong, *Chemistry of Protein Conjugation and Cross-Linking*, CRC Press, London (1991).
8. J. Brunner, "New photolabeling and crosslinking methods," *Ann. Rev. Biochem.* 993, 483–514 (1993).
9. H. Sigrist and P. Zahler, "Selective modification of membrane components," in *The Enzymes of Biological Membranes*, A. Martonosi, Ed., Vol. 1, pp. 333–369, Plenum Press, New York and London (1985).
10. G. Sundarababu and H. Sigrist, "Photoinduced surface immobilization of biomolecules," *Trends Photochem. Photobiol.* 3, 229–241 (1994).
11. P. E. Guire, "Photochemical immobilization of enzymes and other biochemicals," *Meth. Enzymol.* 44, 280–288 (1976).
12. P. E. Guire, "Enzyme immobilization with a thermochemical-photochemical bifunctional agent," U.S. Patent No. 3,959,078 (1976).